Survival and Growth of *Erwinia amylovora* on Apple Leaves

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Abstract

Populations of *Erwinia amylovora* dropped from 10^4/leaf to below detectable limits within 48 hrs on 'Royal Gala' apple shoots inoculated with *E. amylovora* and incubated in a growth chamber at 24°C and high relative humidity (80-95%). Low *E. amylovora* populations (<10/leaf) were detected 6 and 14 days after inoculation. Under orchard conditions in June 2002 and in 2003, *E. amylovora* was detected on leaves after rain events but was short lived. However, in July 2002 *E. amylovora* populations recovered from leaves significantly increased following a thunderstorm that occurred on a hot day (35°C). The day after the storm, on 10 July, low numbers of *E. amylovora* were detected. On 15 July, higher numbers of *E. amylovora* were detected in leaf washings plated on media but *E. amylovora* was not detected in leaf prints on media, suggesting that bacteria were within the leaf. Following 4 cm of rain on 16 July, *E. amylovora* was detected in both washes and prints. The effect of high temperature and rapid temperature change during inoculation were studied under controlled environmental conditions on both 'Royal Gala' and 'M.26' shoots. A post-inoculation incubation temperature of 35°C resulted in more shoot infection than incubation at 24°C. Pre-inoculation incubation temperature did not have a significant effect on shoot infection. Inoculating plants with bacteria at 4°C resulted in more shoot infection than with bacteria at 24°C. When plants were inoculated with cold bacteria (4°C) and incubated at high temperature (35°C), *E. amylovora* quickly became established within young leaves but rapidly declined on the surface of older leaves. Microscopic observation indicated that under these conditions *E. amylovora* would colonize hydathodes and glandular trichomes of young leaves. These results suggest that rapid temperature changes during summer storms can lead to the establishment of *E. amylovora* within the leaf.

INTRODUCTION

Although significant progress has been made in the last 20 years in our ability to predict and control the blossom blight phase of fire blight, the shoot blight phase of the disease remains poorly understood and difficult to control. One of the major gaps in our understanding of shoot blight is the source(s) of inoculum for shoot infection. Although blossom infections are known to be an important source of inoculum for shoot infection (Thomson, 2000), shoot infection is often reported in orchards in the absence of blossom blight (Steiner, 1990b). Epiphytic populations of *Erwinia amylovora* are known to be important in the blossom blight phase of the disease (Thomson, 1986) and predicting the build up of these bacteria in blossoms is a critical component of the MaryBlyt and Cougarblight model (Smith, 1999; Steiner, 1990a). However, there is little data on the epiphytic growth or survival of *E. amylovora* on apple leaves and the role that epiphytic populations play in the shoot blight phase of the disease.

MATERIALS AND METHODS

To monitor populations of *E. amylovora* on orchard grown trees, leaves were collected from 4-yr-old *Malus* x *domestica* (apple) 'Gala' trees on M.26 rootstock (2002) and 14-yr-old 'Golden Delicious' trees on MARK rootstock (2003), with and without fire

blight blossom infections. To distinguish between external and internal populations of the bacteria, leaves were first printed onto CCT (Ishimaru and Klos, 1984) selective medium by briefly placing the leaves on the medium (Thomson, 2000). Leaves were then removed from the medium, washed in 0.05M phosphate buffer (pH 6.8) and washings were plated on CCT. In 2002, assays were generally conducted prior to predicted rainfall and within 24 hours of rain events and in 2003, assays were conducted at time intervals.

In growth chamber studies single-shoot plants of 'Royal Gala' apple and 'Malling 26' (M.26) apple rootstock were grown at 24°C. To study the growth and survival of fire blight bacteria on apple shoots at constant temperature, plants of 'Royal Gala' were spray inoculated with a suspension of *E. amylovora* (10^6 cfu/ml) at 4°C. Plants were incubated in a growth chamber at 24°C, 80-95% relative humidity. At time intervals after inoculation, leaves were harvested and the populations of fire blight bacteria determined by washing leaves in phosphate buffer (0.05 M, pH = 6.5). Individual leaves were washed and washings were plated on CCT medium. Plants were kept in the growth chamber for 30 days and observed for the development of fire blight symptoms.

To study the effect of high temperature and rapid temperature change during inoculation on shoot blight and bacteria survival, M.26 and 'Royal Gala' (replicate experiment) plants were grown at 24°C. Four days prior to inoculation, plants were placed at 24°C or 35°C (pre-inoculation incubation). Plants were then spray inoculated with a suspension of *E. amylovora* (10^6 cfu/ml) at 4°C or 24°C (inoculum temperature). Following inoculation, plants were incubated at 24°C (relative humidity ca. 94%) or 35°C (relative humidity ca. 86%) (post-inoculation incubation) for 30 days. The youngest unfolded leaf (leaf 1) at the time of inoculation and the fifth leaf from the shoot apex (leaf 5) at the time of inoculation were harvested 2 h, 24 h and 132 h after inoculation. Leaves were printed on CCT medium, ground in phosphate buffer and grindates plated on CCT to estimate the population of *E. amylovora* associated with leaves. Plants were observed for the development of fire blight symptoms 22 and 43 days after inoculation.

In microscopic studies, the growth of *E. amylovora* on M.26 apple leaves was observed under two sets of environmental conditions using an *E. amylovora* strain Ea273 (pPROBE-GTkan) labeled with green fluorescent protein (gfp) (Miller et al., 2000), 5 X 10^6 cfu/ml. The environmental conditions compared were: 24°C pre-inoculation incubation, room-temperature inoculum, 24°C post-inoculation incubation; and 35°C pre-inoculation incubation (2 h), 4°C inoculum, 35°C post-inoculation incubation. At various times after inoculation, the first and fifth leaf from the shoot apex at the time of inoculation were observed using a fluorescent stereoscope and a confocal-scanning-laser microscope.

RESULTS

Orchard Monitoring

Under orchard conditions during June 2002, June 2003, and July 2003, *E. amylovora* was detected on leaves after rain events but was short lived. However, in July 2002 *E. amylovora* populations on leaves increased following a thunderstorm that occurred on a hot day (35°C). The day after the storm, 10 July, low numbers of *E. amylovora* were detected on leaves. On 15 July, higher numbers of *E. amylovora* were detected in leaf washings but *E. amylovora* was not detected in leaf prints, suggesting that bacteria were within the leaf. Following 4 cm of rain on 16 July, higher numbers of *E. amylovora* were detected in both washes and prints.

Growth Chamber Experiments: Constant Temperature

When apple shoots were inoculated with the *E. amylovora* and incubated at constant 24°C, bacterial populations dropped from 10^4 cfu/leaf to below detectable limits within 48 hrs. An average of less than 10 cfu/leaf were detected 6 and 14 days after inoculation.

Growth Chamber Experiments: High Temperature

To determine if high temperatures or sudden temperature changes during wetting events could lead to establishment of fire blight bacteria in apple leaves plants were placed in growth chambers at either 24°C or 35°C for approximately 4 days prior to inoculation with the fire blight bacteria. Plants were then spray inoculated with fire blight bacteria which were either warm (24°C) or cold (4°C). After inoculation, half of the plants were incubated at the initial incubation temperature and half of the plants were transferred to the alternate temperature.

A post-inoculation incubation temperature of 35°C resulted in more shoot infection than incubation at 24°C. Pre-inoculation incubation temperature did not have a significant effect on shoot infection. Inoculating plants with bacteria at 4°C resulted in more shoot infection than with bacteria at 24°C.

Plating studies indicated that when plants were incubated at high temperature after inoculation with cold inoculum, *E. amylovora* became established within young leaves. The number of *E. amylovora* recovered 24 hours after inoculation from the youngest leaf of plants incubated at 35°C was higher than that from older leaves. In the young leaves of these plants most bacteria were recovered by plating the ground leaf, not by printing; whereas in older leaves similar numbers of bacteria were recovered by plating and printing. In contrast, on plants inoculated with room-temperature inoculum (24°C) and incubated at 24°C, *E. amylovora* appeared to survive better on the leaf surface but did not become established within the plant.

Microscopy

In general, *E. amylovora* appeared to be a poor epiphyte of the apple leaf surface. Eighteen to 48 hours after inoculation, *E. amylovora* was observed on the surface of both young and mature leaves as single cells, randomly dispersed over the surface of the leaf. Small clumps ("micro"-colonies) of cells could occasionally be observed on young leaves at 24°C.

When plants were inoculated with cold inoculum and incubated at 35°C, *E. amylovora* was found to colonize both hydathodes and glandular trichomes on young leaves at a much higher frequency than similar leaves at constant 24°C (2.5 sites/leaf vs. 0.25 sites/leaf, respectively). At 35°C, *E. amylovora* appeared to have an atypical filamentous form in these structures. *E. amylovora* was not found in association with stomata at either 35°C or 24°C.

At constant 24°C, *E. amylovora* could be found within the leaf laminar in association with small lesions 96 hours after inoculation. The means by which *E. amylovora* had entered the leaf were not apparent.

DISCUSSION

There has been disagreement regarding the epiphytic fitness of *E. amylovora* on leaves and shoots. In the instruction manual for the MARYBLYT program, Steiner and Lightner (1992) suggest that colonization of the leaf surface by *E. amylovora* is part of the normal shoot infection process. However, during discussion at the 9th International Workshop on Fire Blight, several participants expressed the opinion that *E. amylovora* was a relatively poor epiphyte of the leaf surface. Here we report that *E. amylovora* populations rapidly decreased on apple leaves incubated at constant 24°C and high relative humidity, and only low *E. amylovora* populations (<10 cfu/leaf) could be detected 6 and 14 days after inoculation. In addition, we report that observation of the leaf surface with confocal microscopy showed no evidence that *E. amylovora* multiplied on the leaf surface at either 24°C or 35°C. In contrast, Blakeman (1993) found that high populations of *E. amylovora* could persist on *Cotoneaster* plants for more than 24 days when incubated at 24°C and 90% relative humidity. Possible reasons for the difference in our findings include inoculum dose (Blakeman used 10^8 cfu/ml), relative humidity and host species. Blakeman (1993) did report a significant effect of host species on the growth and survival of *E. amylovora* on *Cotoneaster*, *Sorbus* and *Syringa.* Similar to the results

reported here, Mass Geesteranus and de Vries (1984) could not demonstrate multiplication of *E. amylovora* on pear leaves and found that *E. amylovora* on glass coverslips decreased after 1 to 2 days at temperatures greater than 15°C. Under orchard conditions in Utah, Ockey and Thomson (2001, and this workshop) found that *E. amylovora* was spread to nearby leaves with rain and only survived on contaminated leaves for short periods of time (one day). Similar results were obtained in this study monitoring in a West Virginia apple orchard during June 2002 and during the 2003 season. However, in July 2002 we observed an increase in the number of *E. amylovora* recovered from apple leaves that was associated with a temperature decline during a storm.

Rosenberger and VanCamp (2003) have reported that there was a high incidence of immature pear fruit infection during July 2002 in Ulster County, New York. Their data and orchard observations suggest that the cause of these infections were temperature declines during storms or irrigation on days with high temperature. In laboratory studies, Rosenberger and VanCamp (2003) found that when immature pear fruits were incubated at 35°C for 2 hours prior to inoculation with *E. amylovora* inoculum at 21°C or 4°C, 70% and 85% of non-wounded fruit became infected, respectively; compared with 0% infection when non-wounded fruits were incubated at 21°C prior to inoculation with inoculum at 21°C (no temperature decline). They concluded that when immature pear fruits are simultaneously exposed to water, *E. amylovora* and rapid temperature declines fruit infections can develop, and suggested that this may result from thermal contraction of air spaces within fruit. Although we observed that there was a higher incidence shoot infection with cold (4°C) inoculum than with warm (24°C) inoculum in growth chamber studies with apple shoots, there was not a significant effect of pre-inoculation incubation temperature on the incidence of infection. In addition, our observations using confocal microscopy indicated that although *E. amylovora* was present at stomates after inoculation, there was no evidence that *E. amylovora* colonized the air spaces beneath stomata either with or without temperature declines. Rather, on young apple leaves treated with cold inoculum and incubated at high temperature, bacteria were found to colonize hydathodes at the leaf margin and grandular trichomes on the upper surface of the leaf. This suggests that the effect of temperature declines on fruit and leaf infection by *E. amylovora* may differ.

Heald (1915) suggested that leaf infections observed on apple in the area of North Yakama, Washington were the result of hydathode and/or stomata infections due to the initiation and advancement of lesions from the leaf tip or margin and the visible absence of injury. Although detailed meteorological data was not provided, the infections were observed in July following a night shower. Lewis and Goodman (1966) concluded that glandular trichomes and hydathodes on the upper surface of the leaf could serve as natural portals for infection based upon artificial inoculation, without wounding, on the upper margin of young 'Jonathan' apple that resulted in 90% shoot infection in greenhouse tests. Inoculating the lower surface of the leaf or older leaves rarely resulted in infection. Observation of the leaf surface found: the presence of glandular trichomes and no stomates on the upper leaf surface; a high density of leaf hairs and stomates on the lower surface of the leaf, but no glandular trichomes; and that glandular trichomes were lost from older leaves (Lewis and Goodman, 1966). They also reported that leaf infections started at either the lateral or terminal margin of the leaf. In this study, we also observed that most shoot infections occurring at high temperature originated from marginal leaf necrosis (data not shown).

Because *E. amylovora* grows poorly at 35°C in vitro (Paulin, 2000), our observations of a high incidence of apple shoot infection when plants were incubated at 35°C and high relative humidity was unexpected. Perhaps in planta temperature was lower than ambient temperature in growth chambers due to high light intensity and rapid air circulation that would favor a high rate of transpiration and evaporation from the leaf surface.

CONCLUSIONS

These results suggest that rapid temperature changes during summer storms can lead to the establishment of *E. amylovora* within young leaves. Under controlled conditions, *E. amylovora* did not multiply epiphytically on leaves at constant 24°C. However, when plants were inoculated with cold bacteria (4°C) and incubated at high temperature (35°C), *E. amylovora* quickly became established within young leaves but rapidly declined on the surface of older leaves. Microscopic studies indicated that under these conditions (cold inoculum, high post-inoculation incubation temperature), bacteria would colonize hydathodes and glandular trichomes of young leaves. The importance of hydathodes and glandular trichomes colonization to shoot infection is unclear. However, work reported here and in previous studies (Heald, 1915; Lewis and Goodman, 1966) suggests that *E. amylovora* can enter the leaf via hydathodes or glandular trichomes, and that leaf infections resulting from colonization of these structures exhibit marginal leaf necrosis that is distinct from the midrib necrosis typically observed with fire blight.

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